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(54) **Modified protein A.**

(57) Protein A or protein A-like molecules can be coupled to other materials through a single, defined site on the molecule, by the inclusion of a cysteine residue, e.g. by expression of recombinant DNA.

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Description

Modified Protein A

Background of the Invention

Protein A is a cell surface protein found in Staphylococcus aureus. It has the property of binding the Fc region of mammalian antibodies of class IgG, but the affinity varies with host species and antibody subclass. (For a comprehensive review, see Langone, J.J. [1982] Advances in Immunology 32:157-252.) Protein A can be isolated directly from Staphylococcus cell walls, or from the growth media of mutant strains that secrete protein A. In addition, the gene for protein A has been cloned and expressed in Escherichia coli (Lofdahl, S., Guss, B., Uhlen, M., Philipson, L. and Lindberg, M. [1983] Proc. Natl. Acad. Sci. USA 80:697-701; Colbert, D., Anllionis, A., Gelep, P., Farley, J. and Breyer, R. [1984] J. Biological Response Modifiers 3:255-259). This has allowed the production of large amounts of recombinant protein A.

Several applications based on the IgG binding properties of protein A have been developed. These include the following:

Fractionation and purification of antibodies

Protein A has been used for the fractionation of antibodies from sera, and for the purification of monoclonal antibodies. For these purposes, protein A is coupled to a solid matrix such as crosslinked agarose, TRISACRYL (distributed by LKB Instruments, Gaithersburg, MD), or silica-based materials (Langone, J.J. [1982] J. Immunol. Methods 55:277-296).

Therapeutic plasma exchange (TPE)

There is evidence that the removal of circulating immune complexes from blood plasma by their binding to protein A has a therapeutic effect on certain autoimmune and malignant diseases (Salinas, F.A. and Hanna, M.G. [1985] Contemporary Topics in Immunobiology, Plenum Press, NY, Vol. 15, Immune Complexes and Human Cancer). To accomplish this, plasma is passed over a device that consists of protein A attached to an inert, nontoxic support.

Immunochemical procedures

Protein A can be used as a probe for IgG in a number of immunochemical procedures, such as enzymelinked immunosorbant assays (ELISAs). ELISA requires that protein A be coupled to another protein, such as alkaline phosphatase or horseradish peroxidase (Langone, J.J. [1982] J. Immunol. Methods 55:277-296).

Histochemical procedures

Protein A can be used in histochemical or cytological procedures, such as studies of cell surface antigens. For these uses, protein A is often coupled to a fluorescent label, such as by reaction with fluorescein isothiocyanate.

It is clear that from the above discussion that for most uses, protein A must be coupled by covalent bonds to other substances. Although a number of coupling chemistries have been devised, most lead to linkage through a protein A amino group. The exact site of attachment, however, is ambiguous. Protein A contains about fifty amino groups of similar reactivity, and any one or several of these can be involved in coupling. This ambiguity has the following practical disadvantages:

(1) Coupling may occur through an amino group that is required for the antibody binding function of protein A. Even if the group is not involved directly, immobilization through it may disrupt the structure of an Fc binding region.

(2) Protein A may be linked through several sites. Although none of the individual sites are required for antibody binding, coupling through several sites could limit the flexibility of the protein A molecule and thereby reduce its ability to bind antibody.

(3) The coupled product is not homogeneous. Thus, when linked to a solid support, different molecules may have different affinities for antibody, depending on the site of immobilization. This would be disadvantageous for applications such as affinity chromatography where antibodies are separated from contaminating substances. Similarly, fluorescent labeling of protein A will afford a mixture of products. This can lead to irreproducible results in immunoassays.

Brief summary of the Invention

The subject invention concerns a novel protein A or protein A-like molecule that can be coupled to other materials through a single, defined site on the protein A molecule. This protein A or protein A-like molecule is the product of a recombinant protein A gene that has been modified to express a protein containing a single cysteine amino acid residue at a defined position in the amino acid sequence. The novel protein A-like molecule, exemplified herein, is referred to as Cysteinyl-rProtein A (Trademark of Repligen Corporation, Cambridge, MA) (claim 3). The nucleotide sequence encoding Cysteinyl-rProtein A is shown in Chart B.

The protein A gene codes for five antibody binding domains (E, A, B, C, and D) and a C-terminal region (or

"X" region) that does not bind antibodies (Colbert et al, supra). The X region includes amino-acid Glu-310 which is indicated by an asterisk in the claim 3 sequence, and all following amino-acids in that sequence. In accordance with the invention, the gene is modified such that a cysteine residue is expressed in the C-terminal X region. Any one of the antibody-binding regions or a combination thereof can be expressed with the modified C-terminal region to give a protein A-like molecule containing a unique cysteine residue.

Description of the Invention

The invention will be described by way of example only with reference to the accompanying drawings, in which:

Figure A shows the sequence of the procedure used to construct a novel plasmid (pBG3-Cys) that expresses Cysteinyl-rProtein A;

Chart B shows the nucleotide sequence encoding Cysteinyl-rProtein A;

Chart C shows the sequence of a 26-basepair insert; and

Chart D shows a new oligonucleotide duplex inserted into BssHII-restricted pBG3-2ΔN.

Protein A contains no cysteine residues in its amino acid sequence (Colbert et al; supra), but Cysteinyl-rProtein A has been prepared by altering a recombinant protein A gene, expressing the gene in an E. coli host, and purifying the recombinant product.

By way of reference, rProteinA is disclosed and claimed in our European Patent Application No. 88302098.4.

The procedure used to construct the altered protein A gene is outlined in Figure A. Plasmid pBG3-2ΔN contains a 323 bp sequence from an E. coli protein a 1161-bp sequence of the S. aureus protein A gene, a 26-bp synthetic DNA sequence that contains a stop codon for the protein A gene, and a 3722-bp sequence from the well-known plasmid pBR325. The synthetic insert contains two BssHII restriction sites that are found nowhere else in pBG3-2ΔN. The gene was modified by restricting plasmid pBG3-2ΔN with BssHII, and replacing the excised insert with a new synthetic insert containing a codon for a cysteine residue.

Plasmid pBG3-2ΔN, in an E. coli host, is on deposit with the Agricultural Research Culture Collection (NRRL), Northern Regional Research Center, U.S. Department of Agriculture, 1815 North University Street, Peoria, Illinois 61604, USA. The accession number is NRRL B-15910. The plasmid can be removed from this host by standard procedures, for example, by using cleared lysate-isopycnic density gradient procedures, and the like.

The sequence of the new synthetic insert was selected as follows. The sequence of the 26-bp insert in pBG3-2ΔN is shown in Chart C, where the positions of the BssHII restriction sites and the corresponding amino acid sequence are indicated. The C-terminal amino acid residue of the recombinant protein A expressed from pBG3-2ΔN is serine. This residue was replaced by a cysteine residue in Cysteinyl-rProtein A. Because the pKa of the sulfhydryl group of a C-terminal cysteine residue is higher than that of an internal cysteine residue, the terminal group will be less reactive. Therefore, for Cysteinyl-rProtein A, a new glycine residue was inserted C-terminal to the cysteine. However, this glycine residue is not considered to be critical. Further, other amino acids can be adjacent to the cysteine. The desired amino acid sequence of the C-terminal region, and one DNA sequence that will express this, are shown in Chart D. The DNA sequence shown was inserted into the BssHII-restricted plasmid pBG3-2ΔN, and thus replaced the 16-bp BssHII fragment shown in Chart C. Note that the DNA sequence in Chart D is a palindrome. This has the following advantages: The DNA is self-complementary, so only one strand of the inserted duplex need be synthesized; and the synthetic duplex can be inserted in either of the two possible orientations to give the desired DNA sequence. In addition, the DNA sequence shown in Chart D contains two SphI restriction sites. No such sites are found in pBG3-2ΔN, so the presence of the insert in a recombinant molecule can be tested by the ability of restriction endonuclease SphI to cleave the molecule.

Before detailing the construction of the recombinant plasmid that expresses Cysteinyl-rProtein A, the purification of Cysteinyl-rProtein A, and the uses of Cysteinyl-rProtein A, the general methods employed are disclosed.

(1) E. coli strains

All E. coli strains disclosed are E. coli K-12 derivatives. Strains E. coli JM105, E. coli JM103, and E. coli PR13 (F⁻, pnp-13, rna-19, thr-1, leuR6, thi-1, lacY1, xyl-7, mtl-2, malA1, strA132) are well known in the art and can be obtained from known culture repositories or commercial sources. For example, E. coli JM105 has the deposit number NRRL B-18067, and E. coli JM103 has the deposit number NRRL B-39403.

E. coli PR13(pBG3-Cys) has the deposit no. NRRL B-18194 (deposit date 17 March 1987).

(2) E. coli cultures

Cultures were grown in YT medium (8 g tryptone, 5 g yeast extract, and 5 g NaCl per liter). When required, chloramphenicol was added to a concentration of 30 μg/ml. For the preparation of plates, agar was added to the medium to a concentration of 1.5%.

(3) Preparation of plasmid DNA

Plasmids were prepared from E. coli cultures using a modification of the rapid-boiling procedure of Holmes and Quigley (Holmes, D.S. and Quigley, M. [1981], Analytical Biochemistry 114:193-197). Five-ml cultures are grown at 37°C overnight and pelleted. The pelleted cells are resuspended in 0.4 ml of STET buffer (8%

sucrose, 5% TRITON® X-100 [Rohm & Haas Co., Philadelphia, PA] 50 mM Tris(hydroxymethyl)aminomethane-HCl, pH 8.0, 50 mM ethylenediaminetetraacetic acid [EDTA]). Thirty µl of lysozyme (10 mg/ml water) is added and the mixture placed in a boiling water bath for 2 min. The mixture is then centrifuged (10,000 g, 10 min), the solids are removed, and the supernatants treated with an equal volume of isopropanol. After standing at -10°C for 10 min, the solids are pelleted by centrifugation (10,000 g, 15 min) and the supernatants discarded. The pellets are dissolved in 75 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 0.5 mM EDTA) and treated with 75 µl of 7.5 ammonium acetate. After standing for 10 min at 4°C the solids are pelleted by centrifugation (10,000 g, 15 min) and the supernatants removed and treated with 3 volumes of ethanol. After standing for 10 min at -10°C, the precipitated plasmid DNA is pelleted by centrifugation (10,000 g, 15 min), washed with ethanol, and air dried. The pellet is dissolved in 50 µl of TE and stored frozen at -20°C.

(4) Restriction endonuclease digestions

Restriction endonuclease digestions were performed using the procedures recommended by the manufacturer. The buffer used was 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 75 mM NaCl, and 100 µg/ml bovine serum albumin.

(5) Electrophoretic separation of DNA fragments

Restriction fragments were separated by electrophoresis on 1% agarose gels in TBE buffer (90 mM Tris base, 0.89 M boric acid, 2 mM EDTA) containing 0.5 µg/ml ethidium bromide. Fragments were visualized by illumination with ultraviolet light and their sizes measured by reference to fragments of known size.

(6) Preparation of competent E. Coli cells

Cultures of E. coli were grown at 37°C with agitation until the absorbance at 600 nm was 0.3. The cells were then chilled on ice, pelleted by centrifugation (4100 g, 10 min), resuspended in 1/2 the original volume of ice-cold 50 mM $CaCl_2$, and incubated on ice for 20 min. The cells were collected by centrifugation as above and resuspended in 1/25 the original volume of ice-cold 50 mM $CaCl_2$. One-ml aliquots were stored frozen at -80°C.

(7) Transformation of competent cells

Frozen competent cells were thawed and 0.2 ml were treated with approximately 0.4 µg of plasmid DNA in 5-20 µl of TE. After standing for 30 min on ice, the mixture was placed in a 37°C water bath for 2 min and then treated with 1 ml of YT medium and incubated 1 h at 37°C. The cultures were then plated on YT medium containing 30 µg/ml chloramphenicol and grown at 37°C.

(8) Polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was performed on polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) as described by Laemmli (Laemmli, U.K. [1970] Nature [London] 227:680-685). Slab gels were 1.5 mm thick and contained a total acrylamide concentration of 12%. Samples (up to 25 µl) were mixed with 25 µl of sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.0025% bromphenol blue), placed in a boiling water bath for 2 min, cooled, and loaded on the gel. Electrophoresis was performed at 75 mA in an apparatus purchased from Hoeffer Scientific Instruments (San Francisco, CA). Gels were stained with a solution of 0.5 g/l coomassie blue in 5:5:1 methanol/water/acetic acid and destained in 7.5% acetic acid.

(9) Synthesis and phosphorylation of oligodeoxyribonucleotide

The oligonucleotide dCGCGCATGCGGCTAGCCGCATG was synthesized using an Applied Biosystems (Foster City, CA) model 380A DNA Synthesizer using the phosphoramidite procedure recommended by the manufacturer. The deprotected oligomer was purified using the electrophoretic method of Atkinson and Smith (Atkinson, T. and Smith, M. [1984] in Oligonucleotide Synthesis: A Practical Approach, Gait, M.J., Ed. IRL Press, Arlington, VA, pp. 35-81.) The purified oligomer (29 µg) was treated with 10 units of T4 polynucleotide kinase in 30 µl of 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.1 mM spermidine, 0.1 mM EDTA, and 0.15 M adenosine triphosphate (ATP). The solution was incubated for 30 min at 37°C and then the DNA was precipitated with 1/10 volume of 3 M sodium acetate, pH 4.7, and 3 volumes of ethanol (-10°C, 10 min). The DNA was pelleted by centrifugation (10,000 g, 15 min), washed with ethanol, and dried. The pellet was redissolved in 30 µl of TE, heated to 60°C, and cooled slowly to room temperature in order to form a DNA duplex.

Following are examples which illustrate procedures for practising the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1—BssHII endonuclease digestion of plasmid pBG3-2ΔN

One µg of pBG3-2ΔN was incubated with 12 units of BssHII in the buffer described above in (4) for 120 min at 37°C. One µl (20 units) of alkaline phosphatase (calf intestine) was added and the solution incubated for an additional 1 h at 37°C. The solution was then diluted to 100 µl with TE, extracted with two 50-µl portions of phenol, extracted with two 100-µl portions of diethyl ether, treated with 1/10 volume of 3 M sodium acetate (pH

4.7), and precipitated with 3 volumes of ethanol. The restricted, phosphorylated DNA was pelleted by centrifugation (10,000 g, 15 min), washed with ethanol, dried, and dissolved in 30 µl TE.

Example 2--Ligation of oligonucleotide insert

The dephosphorylated BssHII restriction fragments of pBG3-2ΔN (0.5 µg), the synthetic oligonucleotide duplex described in (9) (5 µg), and T4 DNA ligase (400 units) were incubated in 50 mM Tris-HCl, pH 7.8, 6 mM $MgCl_2$, 20 mM DTT containing 1 mM ATP for 15 h at 16°C. The reaction mixture was used to transform competent E. coli JM105 cells as described above in (7).

Example 3--Screening of transformants for new SphI restriction sites

Plasmid DNA was isolated from 10 colonies of transformants from Example 2. This DNA was restricted by a mixture of SphI and EcoRI. Agarose electrophoresis revealed the fragments that would be expected from the insertion of the DNA sequence shown in Chart D into the BssHII restriction fragment of pBG3-2ΔN, namely, one of 1.2 kbp and one of 4.0 kbp. Plasmid DNA from all 10 of the transformants tested gave these fragments on digestion. By contrast, plasmid pBG3-2ΔN gave, as expected, only a single fragment of 5.2 kbp. The plasmid isolated from the transformants was designated pBG3-Cys.

Example 4--Transformation of E. Coli PR13 with pBG3-Cys

Plasmid pBG3-Cys from one of the transformants described in Example 3 was used to transform competent E. coli JM103 cells. Plasmid isolated from 4 of the E. coli JM103(pBG3-Cys) transformants was screened for the SphI site as described in Example 3, and all were found to contain it. Plasmid from 1 of these transformants was used to transform competent E. coli PR13 cells. Plasmid was isolated from 4 of the E. coli PR13(pBG3-Cys) transformants and screened for the presence of the SphI site. All 4 were found to contain the site.

Example 5--Expression of Cysteinyl-rProtein A by E. coli PR13(pBG3-Cys)

Cultures of E. coli PR13(pBG3-Cys) and E. coli PR13(pBG3-2ΔN) were grown overnight at 37° C and 50 µl of each was pelleted. The pellets were suspended in 25 µl of sample buffer and subjected to SDS-PAGE as described in (8). The destained gel showed that the E. coli PR13(pBG3-Cys) cultures express a protein of the same apparent molecular weight and in the same amount as the recombinant protein A known to be expressed by E. coli PR13(pBG3-2ΔN).

Example 6--Large-scale fermentation of E. coli PR13(pBG3-Cys)

Ten ml of an overnight culture of E. coli PR13(pBG3-Cys) in YT containing 30 µg/ml chloramphenicol was used to inoculate 10 L of modified 2% medium (20 g/L yeast extract, 20 g/L casamino acids, 20 g/L casein peptone, 2 g/L K_2HPO_4, 2 g/L KH_2PO_4, 2 g/L $Na_2HPO_4.7H_20$) containing 10 mg/L chloramphenicol in a 20l Chemapec fermenter (Chemapec, Inc., Woodbury, NY). The recombinant cells were grown at 37° C with 50% dissolved O_2. The pH was maintained at 6.81 with 85% lactic acid and 50% NaOH. Foam was controlled by the addition of ANTIFOAM B (E.I. DuPont de Nemours & Co., Inc., Wilmington, DE). Cells were harvested in late log phase by centrifugation (3,500 xg, 20 min). Cell yields were 30 g wet cell weight (wcw) per liter.

Example 7--Lysis of recombinant cells

Harvested E. coli PR13(pBG3-Cys) cells (150 g) were suspended in 1 L of lysis buffer (10 mM Tris-HCl, pH 8.3, 5 mM EDTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], and 0.5% TRITON® X-100). Lysis was performed by passing the suspension 2 times through a Dynomill model KDL-Pilot (Impandex, Maywood, NJ) charged with 1.2 L of glass beads (0.5-0.7 mm diameter). The lysate was clarified by centrifugation (7,310 g, 45 min).

Example 8--Purification of Cysteinyl-rProtein A

An 11x13 cm column of DEAE-SEPHAROSE FAST FLOW (Pharmacia Fine Chemicals, Piscataway, NJ) was equilibrated with wash buffer (35 mM Tris-HCl, pH 8.3, 2 mM EDTA, 1 mM DTT, 0.1 mM PMSF) and 1.5 L of clarified lysate (Example 7) was passed over the column at a flow rate of 100 ml/min. Wash buffer (3 L) was then passed over the column at 100 ml/min. The flow rate was then reduced to 10 ml/min, and Cysteinyl-rProtein A was eluted by a linear gradient formed from 4 L of wash buffer and 4 L of elution buffer (wash buffer containing 200 mM KCl). Two-hundred-ml fractions were collected, and samples from each fraction were analyzed by SDS-PAGE. Those containing a protein of 40 kilodalton (kd) apparent molecular weight (Cysteinyl-rProtein A) were pooled and heated to 85°C for 10 min. Precipitated contaminants were pelleted by centrifugation (7,310 g, 30 min) and the supernatant was passed through a 0.45-micron filter. The filtrate was treated with 3 volumes of ethanol and stirred for 30 min at room temperature. The resulting precipitate was collected by centrifugation (7,310 g, 30 min) and dried. The pellet was dissolved in sterile TE and stored at 4°C in the presence of 1 mM DTT. The 40 kd product was judged to be >90% pure by SDS-PAGE. The yield, as measured by absorbance at 275 nm, was 5 mg per g wcw.

Example 9—Fluorescein-labeled protein A

The reagent fluorescein-5-maleimide (available from Molecular Probes, Inc., Eugene, OR) reacts with sulfhydryl groups of proteins to form stable covalent bonds. Cysteinyl-rProtein A (28 µg) and fluorescein-5-maleimide (16 µg) were incubated in 25 µl of 0.1 M sodium phosphate, pH 7.5, 0.5 mM EDTA for 30 min at 37°C. The solution was subjected to SDS-PAGE as described above (8) and the fluorescent products visualized by illumination with ultraviolet light before coomassie staining. This revealed an intensely fluorescent protein of 40 kd molecular weight corresponding to Cysteinyl-rProtein A. Under the same conditions, recombinant protein A from E. coli PR13(pBG3-2ΔN) showed a product with little or no fluorescence.

The product of the reaction of Cysteinyl-rProtein A and fluorescein-5-maleimide was passed over a column of human IgG immobilized to agarose (Mayes, E.L.V. [1984] in Methods in Molecular Biology, Volume 1, Proteins, Walker, J.M. Ed. Humana Press, Cliffton, NJ, pp. 13-20). The fluorescent product was bound by the column and not eluted by extensive washing with phosphate-buffered saline (PBS). The fluorescent protein was eluted by 0.2 M glycine, pH 2.0, as would be expected for a protein A-like material. A 40 kd, fluorescent protein was revealed by SDS-PAGE of the column eluate.

Example 10—Coupling of Cysteinyl-rProtein A to thiol affinity materials

ACTIVATED THIOL SEPHAROSE 4B (Pharmacia Fine Chemicals) is a gel that reacts with reduced sulfhydryl groups to form stable, covalent disulfide bonds. Cysteinyl-rProtein A (2 mg) in 0.5 ml of 0.1 M sodium phosphate, pH 7.5, 0.5 mM EDTA was mixed with 0.5 ml of swelled ACTIVATED THIOL SEPHAROSE 4B and turned for 2 h at room temperature. The gel was washed with PBS until no protein was observed in the wash. The gel was then treated with 10 mM DTT in order to reduce the disulfide bonds and thereby release the bound proteins. Approximately 1 mg of a 40 kd protein was liberated per ml of gel, as measured by SDS-PAGE. When the experiment was performed using the recombinant protein A expressed by E. coli PR13(pBG3-2ΔN), no protein was released from the gel by DTT treatment.

The nucleotide sequence encoding Cysteinyl-rProtein A can be prepared by a "gene machine" by procedures well known in the art. This is possible because of the disclosure of the nucleotide sequence.

Cysteinyl-rProtein A can be chemically synthesized by solid phase peptide synthetic techniques such as BOC and FMOC (Merrifield, R.B. [1963] J. Amer. Chem. Soc. 85:2149; Chang, C. and Meinenhofer, J. [1978] Int. J. Peptide Protein Res. 11:246).

As is well known in the art, the amino acid sequence of a protein is determined by the nucleotide sequence of the DNA. Because of the redundancy of the genetic code, i.e., more than one coding nucleotide triplet (codon) can be used for most of the amino acids used to make proteins, different nucleotide sequences can code for a particular amino acid. Thus, the genetic code can be depicted as follows:

Phenylalanine (Phe)	TTK	Histidine (His)	CAK
Leucine (Leu)	XTY	Glutamine (Gln)	CAJ
Isoleucine (Ile)	ATM	Asparagine (Asn)	AAK
Methionine (Met)	ATG	Lysine (Lys)	AAJ
Valine (Val)	GTL	Aspartic acid (Asp)	GAK
Serine (Ser)	QRS	Glutamic acid (Glu)	GAJ
Proline (Pro)	CCL	Cysteine (Cys)	TGK
Threonine (Thr)	ACL	Tryptophan (Trp)	TGG
Alanine (Ala)	GCL	Arginine (Arg)	WGZ
Tyrosine (Tyr)	TAK	Glycine (Gly)	GGL
Termination signal	TAJ		

Key: Each 3-letter deoxynucleotide triplet corresponds to a trinucleotide of mRNA, having a 5'-end on the left and a 3'-end on the right. All DNA sequences given herein are those of the strand whose sequence corresponds to the mRNA sequence, with thymine substituted for uracil. The letters stand for the purine or pyrimidine bases forming the deoxynucleotide sequence.

A = adenine
G = guanine
C = cytosine
T = thymine
X = T or C if Y is A or G
X = C if Y is C or T
Y = A, G, C or T if X is C
Y = A or G if X is T
W = C or A if Z is A or G
W = C if Z is C or T
Z = A, G, C or T if W is C
Z = A or G if W is A
QR = TC if S is A, G, C or T; alternatively,
QR = AG is S is T or C
J = A or G
K = T or C
L = A, T, C or G
M = A, C or T

The above shows that the novel amino acid sequence of the subject invention can be prepared by nucleotide sequences other that that disclosed herein. Functionally equivalent nucleotide sequences encoding the novel amino acid sequence of Cysteinyl-rProtein A, or fragments thereof having protein A-like activity, can be prepared by known synthetic procedures. Accordingly, the subject invention includes such functionally equivalent nucleotide sequences.

In addition it has been shown that proteins of identified structure and function may be constructed by changing the amino acid sequence if such changes do not alter the protein secondary structure (Kaiser, E.T. and Kezdy, F. J. [1984] Science 223:249-255). Thus the subject invention includes mutants of the amino acid sequence depicted herein which do not alter the protein secondary structure, or if the structure is altered, the biological activity is retained to some degree.

As shown above, it is well within the skill of those in the genetic engineering art to use the nucleotide sequence encoding Cysteinyl-rProtein A activity of the subject invention to produce proteins via microbial processes. Fusing the sequences into an expression vector and transforming or transfecting into hosts, either eukaryotic (yeast or mammalian cells) or prokaryotic (bacterial cells), are standard procedures used in producing other well-known proteins, e.g., insulin, interferons, human growth hormone, and the like. Similar procedures, or obvious modifications thereof, can be employed to prepare Cysteinyl-rProtein A of the subject invention by microbial means or mammalian tissue culture technology. Further, the antibody-binding domains of protein A can be prepared by standard gene machine procedures. These domains can be used individually or in various combinations with the X-region, disclosed herein, fused thereto. Modification of the X region to code for a cysteine residue can be done before or after fusion to the domain(s) by standard procedures.

The protein A gene also can be modified so as to incorporate a cysteine in the N-terminal coding sequence preceding the IgG binding domain(s). Alternatively, an N-terminal cysteine can be further removed from the IgG binding domain(s) by incorporating a synthetic DNA sequence at the 5' end of the protein A gene to create a polypeptide "spacer" between the cysteine and the IgG binding domain(s). This spacer could be about 1 to about 100 amino acids in length. As disclosed herein, the modification can be made to any of the protein A domains and the domains can be used individually or in various combinations with the modified region fused thereto. These modifications, wherein the cysteine residue is outside the IgG binding regions, can be done readily by a person skilled in the art using standard procedures.

Claims

1. Modified protein A, or a part including one or more (in combination) of the E,A,B,C and D domains thereof, the modification comprising a cysteine residue outside the IgG binding region.

2. Modified protein A, or domain(s) thereof, according to claim 1, wherein the cysteine residue is in the X region.

3. Modified protein A according to claim 1, having the following amino-acid sequence:

Met Leu Arg Pro Val Glu Thr Pro Thr Arg Glu Ile Lys Lys
Leu Asp Gly Leu Ala Gln His Asp Glu Ala Gln Gln Asn Ala
Phe Tyr Gln Val Leu Asn Met Pro Asn Leu Asn Ala Asp Gln
Arg Asn Gly Phe Ile Gln Ser Leu Lys Asp Asp Pro Ser Gln
Ser Ala Asn Val Leu Gly Glu Ala Gln Lys Leu Asn Asp Ser
Gln Ala Pro Lys Ala Asp Ala Gln Gln Asn Lys Phe Asn Lys
Asp Gln Gln Ser Ala Phe Tyr Glu Ile Leu Asn Met Pro Asn
Leu Asn Glu Glu Gln Arg Asn Gly Phe Ile Gln Ser Leu Lys
Asp Asp Pro Ser Gln Ser Thr Asn Val Leu Gly Glu Ala Lys
Lys Leu Asn Glu Ser Gln Ala Pro Lys Ala Asp Asn Asn Phe
Asn Lys Glu Gln Gln Asn Ala Phe Tyr Glu Ile Leu Asn Met
Pro Asn Leu Asn Glu Glu Gln Arg Asn Gly Phe Ile Gln Ser
Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu Ala Glu
Ala Lys Lys Leu Asn Glu Ser Gln Ala Pro Lys Ala Asp Asn
Lys Phe Asn Lys Glu Gln Gln Asn Ala Phe Tyr Glu Ile Leu
His Leu Pro Asn Leu Asn Glu Glu Gln Arg Asn Gly Phe Ile
Gln Ser Leu Lys Asp Asp Pro Ser Gln Ser Ala Asn Leu Leu
Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro Lys Ala
Asp Asn Lys Phe Asn Lys Glu Gln Gln Asn Ala Phe Tyr Glu
Ile Leu His Leu Pro Asn Leu Thr Glu Glu Gln Arg Asn Gly
Phe Ile Gln Ser Leu Lys Asp Asp Pro Ser Val Ser Lys Glu
Ile Leu Ala Glu Ala Lys Lys Leu Asn Asp Ala Gln Ala Pro
Lys Glu Glu Asp Asn Asn Lys Pro Gly Lys Glu Asp Gly Asn
Lys Pro Gly Lys Glu Asp Gly Asn Lys Pro Gly Lys Glu Asp
Asn Lys Asn Leu Gly Lys Glu Asp Gly Asn Lys Pro Gly Lys
Glu Asp Asn Lys Lys Pro Gly Lys Glu Asp Gly Asn Lys Pro
Gly Lys Glu Asp Gly Asn Lys Pro Gly Lys Glu Asp Gly Asn
Lys Pro Gly Lys Glu Asp Gly Asn Lys Pro Gly Lys Glu Asp
Gly Asn Lys Pro Gly Lys Glu Asp Gly Asn Gly Val Ile Gly
Arg Ala Cys Gly

4. The gene for modified protein A, or domain(s) thereof, according to any preceding claim.

5. A DNA transfer vector comprising DNA coding for modified protein A, or domain(s) thereof, according to any of claims 1 to 3.

6. Plasmid pBG3-Cys, a transfer vector according to claim 5.

7. A host into which a DNA transfer vector according to claim 5 or claim 6 has been transferred and replicated.

8. E. coli PR13(pBG3-Cys), NRRL B-18194, a host according to claim 7.

9. A process for preparing modified protein A, or domain(s) thereof, according to any of claims 1 to 3, which comprises culturing a microbial host transformed with a DNA transfer vector according to claim 5 or claim 6.

10. A process selected from

(a) fractionating antibodies from sera;

(b) purifying monoclonal antibodies;

(c) removing circulating immune complexes from blood plasma;

(d) probing for IgG in immunochemical procedures; and

(e) histochemical or cytological procedures,

which comprises covalent coupling of modified protein A according to any of claims 1 to 3.

11. A process according to claim 10, wherein the modified protein A is fluorescein-labelled.

12. A process according to claim 10, wherein the modified protein A is coupled to a thiol affinity material.

Figure A

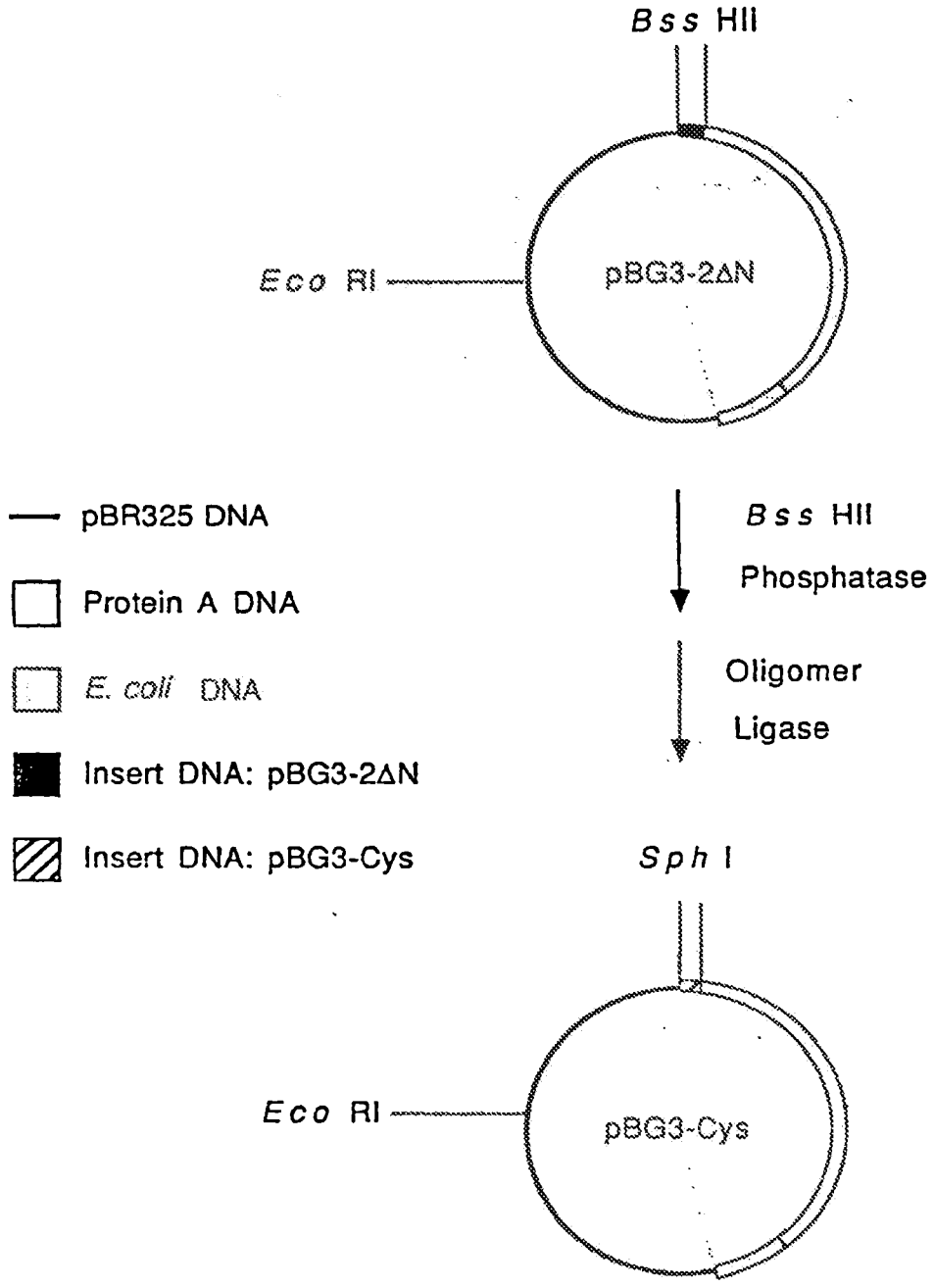
Bss HII
Eco RI
pBG3-2ΔN
pBR325 DNA
Protein A DNA
E. coli DNA
Insert DNA: pBG3-2ΔN
Insert DNA: pBG3-Cys
Bss HII
Phosphatase
Oligomer
Ligase
Sph I
Eco RI
pBG3-Cys

Chart B

CATATGTCAT GAGAGTTTAT
CGTTCCCAAT ACGCTCGAAC GAACGTTCGG TTGCTTATTT TATGGCTTCT
GTCAACGCTG TTTTAAAGAT TAATGCGATC TATATCACGC TGTGGGTATT
GCAGTTTTTG GTTTTTTGAT CGCGGTGTCA GTTCTTTTTA TTTCCATTTC
TCTTCCATGG GTTTCTCACA GATAACTGTG TGCAACACAG AATTGGTTAA
CTAATCAGAT TAAAGGTTGA CCAGTATTAT TATCTTAATG AGGAGTCCCTT
ATG
TTA CGT CCT GTA GAA ACC CCA ACC CGT GAA ATC AAA AAA CTC
GAC GGC CTT GCG CAA CAC GAT GAA GCT CAA CAA AAT GCT TTT
TAT CAA GTG TTA AAT ATG CCT AAC TTA AAC GCT GAT CAA CGT
AAT GGT TTT ATC CAA AGC CTT AAA GAT GAT CCA AGC CAA AGT
GCT AAC GTT TTA GGT GAA GCT CAA AAA CTT AAT GAC TCT CAA
GCT CCA AAA GCT GAT GCG CAA CAA AAT AAG TTC AAC AAA GAT
CAA CAA AGC GCC TTC TAT GAA ATC TTG AAC ATG CCT AAC TTA
AAC GAG GAG CAA CGC AAT GGT TTC ATT CAA AGT CTT AAA GAC
GAT CCA AGC CAA AGC ACT AAC GTT TTA GGT GAA GCT AAA AAA
TTA AAC GAA TCT CAA GCA CCG AAA GCT GAC AAC AAT TTC AAC
AAA GAA CAA CAA AAT GCT TTC TAT GAA ATC TTG AAC ATG CCT
AAC TTG AAC GAA GAA CAA CGC AAT GGT TTC ATC CAA AGC TTA
AAA GAT GAC CCA AGT CAA AGT GCT AAC CTT TTA GCA GAA GCT
AAA AAG TTA AAT GAA TCT CAA GCA CCG AAA GCT GAT AAC AAA
TTC AAC AAA GAA CAA CAA AAT GCT TTC TAT GAA ATC TTA CAT
TTA CCT AAC TTA AAT GAA GAA CAA CGC AAT GGT TTC ATC CAA
AGC TTA AAA GAT GAC CCA AGC CAA AGC GCT AAC CTT TTA GCA
GAA GCT AAA AAG CTA AAT GAT GCA CAA GCA CCA AAA GCT GAC
AAC AAA TTC AAC AAA GAA CAA CAA AAT GCT TTC TAT GAA ATT
TTA CAT TTA CCT AAC TTA ACT GAA GAA CAA CGT AAC GGC TTC
ATC CAA AGC CTT AAA GAC GAT CCT TCA GTG AGC AAA GAA ATT
TTA GCA GAA GCT AAA AAG CTA AAC GAT GCT CAA GCA CCA AAA
GAG GAA GAC AAC AAC AAG CCT GGT AAA GAA GAC GGC AAC AAA
CCT GGT AAA GAA GAC GGC AAC AAA CCT GGT AAA GAA GAC AAC
AAA AAC CTT GGC AAA GAA GAC GGC AAC AAA CCT GGT AAA GAA
AAA GAA GAC GGC AAC AAG CCT GGT AAA GAA GAT GGC AAC AAA
CCT GGT AAA GAA GAT GGC AAC AAG CCT GGT AAA GAA GAT GGC
AAC AAG CCT GGT AAA GAA GAC GGC AAC GGA GTC ATC GGG CGC
GCA TGC GGC TAG CCGCATGCGCGCCCG

Chart C

```
     Gly Arg Ala Ser Stop
5' C GGG CGC GCT AGC TAG CTA GCG CGC C 3'

3' G CCC GCG CGA TCG ATC GAT CGC GCG G 5'
          Bss HII             Bss HII
```

Chart D

```
     Arg Ala Cys Gly Stop
5'   CGC GCA TGC GGC TAG CCG CAT G   3'

3'       GT ACG CCG ATC GGC GTA CGC GC 5'
         Sph I             Sph I
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European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 88 30 2561

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	EP-A-0 107 509 (REPLIGEN CORPORATION)		C 12 N 15/00 C 12 P 21/02 C 12 N 1/20 C 07 K 13/00 C 07 K 17/00 G 01 N 33/58 // (C 12 N 1/20 C 12 R 1:19)
P,A	WO-A-8 702 987 (J.R. MURPHY)		

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

C 12 N
C 12 P

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	27-06-1988	VAN PUTTEN A.J.

CATEGORY OF CITED DOCUMENTS

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